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Title

Agro-waste utilization for the production of therapeutic enzymes from microbial isolates and their application.

Abstract

Microbial therapeutic enzymes are of greater importance as these are used in the treatment of different chronic diseases like Cancer or leukemia, skin ulcers, cardiovascular diseases, pancreatic disorders, digestive disorders, Parkinson's disease, Pompe's disease, and Fabry's disease. These enzymes have maximum specificity, affinity, and efficiency. Therapeutic enzymes have wide applications in the medical field and food industries. In the food industry for increase the shelf life of the food material by converting hydrogen peroxide into oxygen molecules. They are required for many chemical reactions during the metabolism of individuals. These are also called biocatalysts which enhance the rate of reaction during metabolism. These help to treat diseases that cannot be cured by conventional treatment, so therapeutic enzymes are getting more importance day by day. The present work will compile all the information of some therapeutic enzymes and their sources, microbial cultures, methods for the production of therapeutic enzymes, and applications with special emphasis on L-asparaginase, streptokinase, and glutaminase. It is an attempt to assess the current research related to production of these therapeutic enzymes by using agricultural waste as a substrate and advancements in therapeutic enzymes for the improvement of new enzymes and its applications in the medical field.

Keywords: Agricultural waste substrate; therapeutic enzymes; leukemia; biocatalyst

Introduction

In recent years, therapeutic enzymes have gained great importance in clinical research, pharmaceutical research, development, and manufacturing. Therapeutic enzymes have been in use for at least 40 years. This new advancement in pharmaceutical science is intended to cure diseases that may not be curable by conventional treatment methods.

Enzymes play a vital role in maintaining good health and good metabolism. Each cell requires enzymes for proper functioning as well as for appropriate biochemical functions. Enzymes are biocatalysts so they can accelerate the rate of reaction in the body and maintain the regulatory system properly. India is also called as Agricultural nation. It is a resource center for collecting agricultural waste. According to the report it is observed that 1500 lakh ton agro waste produced in India per year. So agricultural waste is a good substrate for the fermentation process for getting valuable products.

Different agricultural waste like corn cob, wheat bran, rice bran, sugarcane bagasse, groundnut oil cake, black gram husk, red gram husk are commonly used substrates for the production of Asparaginase, L-glutaminase and streptokinase enzyme.

Review of Literature

Bioprocess is one of the key processes that helps in enhancing metabolite productivity under a given set of fermentation environments (Prakasham *et al.* 2007a). For effective and economic process development, investigation of renewable energy sources as solid fermentation medium will certainly help in the eco-friendly treatment of bio-waste with value addition (Pandey 1994; Venkateswarlu et *al*. 2000; Srinivas *et al*. 2003; Prakasham *et al*. 2007b) and submerged fermentation also. Several studies reported that the use of agro-industrial waste materials improved microbial product production with the supplementation of agro-material-specific deficient nutrient sources (Prakasham *et al.* 2006; Rispoli and Shah 2007).

In particular, glutaminase production has been reported under solid-state fermentation using wheat bran, sesame oil cake and polystyrene beads (Prabhu and Chandrasekaran 1997; Sabu *et al*. 2000a; Kashyap *et al*. 2002). To date, maximum glutaminase production of 49 890 U l) 1 was reported under solid-state fermentation by Beauveria sp. BTMFS 10 (Sabu *et al.* 2000b), while 410 U l) 1 was reported under submerged fermentation by Zygosaccharomyces rouxii (Iyer and Singhal 2007).

L-asparaginase (L-asparagine amidohydrolase E.C.3.5.1.1) is an enzyme belonging to the amidase group, which catalyzes the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia (Gurunathan and Sahadevan, 2012). Industrial production of L-asparaginase by fungi throughout the world is carried out mainly by submerged fermentation (SmF) (Meghavarnam and Janakiraman, 2017; Kumar *et al*., 2013). Solid-state fermentation (SSF) has the potential for the production of enzymes and has emerged as an attractive alternative to submerged fermentation. SSF has attained much relevance during the past decade to offer potential environmental benefits, as it utilizes low-cost agro-industrial residues as the substrate, which is attractive for bioprocessing (Thomasms *et al*., 2013).

Streptokinase (SK) is a therapeutic enzyme, which in simple terms, is essential to break down blood clots. It is a thrombolytic enzyme with a molar mass of 47 kDa (415 amino acid residues) which shows maximum activity at a pH of 7.5 (isoelectric pH is 4.7) . It is a single-chain polypeptide, which forms a complex with the plasminogen activator, which in turn converts the precursor (plasminogen) to plasmin. Plasmin further degrades the fibrin (fibrinolysis). Streptokinase is still a significant agent in the treatment of cardiovascular diseases. Streptokinase, being so economical, has an important value in treating cardiac diseases in developing countries.

- **Aim and Objectives:-**

The present research will aim to produce Asparaginase, L-glutaminase and streptokinase

enzymes using locally available agro-industrial residues by fermentation by selected fungi and bacteria. This will optimize it in a way that will facilitate and encourage future large-scale local production of these therapeutic enzymes in cost cost-effective manner and can be used in the medical field. The work will encompass the following objectives:

- To isolate and characterize suitable bacteria and fungi from soil and water samples.
- Identify the best substrate from various agro-industrial residues for the production of these therapeutic enzymes.
- Optimization of fermentation conditions for the production of therapeutic enzymes with the best substrate using selected fungi and bacteria.
- To investigate the growth kinetics, cost-effectiveness, and therapeutic enzyme production of selected fungi and bacteria.
- To characterize and identify the isolated microbial species by 16s RNA sequencing.
- To perform molecular docking for finding the best binding sites or locations to an enzyme surface.
- Possible objective- To study these produced therapeutic enzymes against the appropriate cell line.

Significance of this study

- This study can provide the good agro waste utilization for the production of different therapeutic enzymes.
- It is most economical method for production of these enzymes.
- For treating the diseases which are not cure by conventional methods will be treat by using these enzymes and it will be cheaper in cost.

Research Methodology

- **Isolation and Screening of fungi and bacteria for production of therapeutic enzymes:-**

Soil and water samples will be collected and stock solution is prepared by adding 1gm soil in 99 ml distilled water and centrifuge it at 2000 rpm for 5 min and then prepare serial dilutions of it. Then each dilution is streak on Minimal Glutamine agar medium. Those colonies associated with pink zone after incubation is considered as the test bacteria and fungi.

For isolating asparaginase producing bacteria the dilutions of soil and water are streaked on M9 media after incubation those colonies forming pink zone is selected for next large scale production.

For streptokinase enzyme bacterial identification these colonies grow on the blood agar those cells after incubation forming discoloration around them and that cells will be selected for production of the enzyme.

- **Preparation of fermentation substrates**

Agricultural waste residues which are abundantly available in vicinity at regional level like corn cob, wheat bran, rice bran, sugarcane bagasse, groundnut oil cake, black gram husk, red gram husk will be procured from nearby area of Ch. Smabhaji Nagar and Parbhani district and are used as solid substrates for therapeutic enzymes production by fermentation.

- **Pre-treatment of Substrates**

All these substrates were powdered and dried in a hot air oven overnight at 60°C. The solid particles were sieved using standard sieves and the particles 1.4 mm were stored in air-tight containers for further use (Prabhu and Chandrasekaran, 1996).

- **Production of Therapeutic enzymes by fermentations**

Using above mentioned substrates and fermentation procedure is performed.

- **Assaying for Therapeutic enzyme**

L-glutaminase:- It can be perfomed by calculating the liberation of NH3 in phosphate buffer and in the presence of L- glutamin.

Asparaginse :- Modified Czapek Dox's medium was supplemented with 0.3 ml of 2.5% phenol red dye prepared in ethanol at pH 6.5 with l-asparagine incorporated in the medium for evaluation of lasparaginase activity. The media was autoclaved and the plates were inoculated with 3 day old culture of fungal isolates. The clear zone appeared after 48 h of growth. Uninoculated media served as control.

Stereptokinase:- Assay Streptokinase assays depend on its ability to activate plasminogen to plasmin. Plasmin hydrolyzes the indicator substrate and increased the hydrolysis at given period which is related back to streptokinase concentration.

- **Optimization of parameters for therapeutic enzyme production**

Effect of pH, temperature, incubation time, substrate concentration, moisture content, particle size etc. will be optimized for high enzyme titers. Higher level production kinetics and cost estimates will be attempted for prediction on the basis of findings.

- **Biomass monitoring**

Biomass during fermentations will be monitored by an indirect procedure, which turned out to be simple and quick (Ruiz *et al*., 2012).

- **Format:**

I intend to structure my thesis as a series of Chapters which roughly will be as follow: Abstract

CHAPTER 1 INTRODUCTION

1.1 Introduction

1.2 Aims and Objectives

1.3 Significance of the study

CHAPTER 2 LITERATURE REVIEW

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2.1.1 Substrate

2.1.2 Bioreactor for SSF and SMF

2.1.3 Factors Affecting Fermentation

- Type of microorganism
- Inoculum Size
- Substrate
- Temperature
- Moisture Content and Water Activity
- pH
- Particle Size of the Substrate
- Agitation

2.1.4 Advantages of SSF and SMF

2.1.5 Disadvantages of SSF and SMF

2.2 Therapeutic enzymes

2.2.1 Asparaginase enzymes

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2.2.3 Sources of asperaginase.

2.2.4 Production of asperaginase.

2.2.5 Applications of asperaginase.

2.2.6 Global asperaginase market

2.2.7 L-glutaminase enzyme

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2.2.9 Sources of L-glutaminase

2.3.0 Production of L-glutaminase

2.3.1 Applications of L-glutaminase.

2.3.2 Global L-glutaminase market

2.3.3 Streptokinase enzyme

2.3.4 Mechanism of action of streptokinase enzyme

2.3.5 Sources of streptokinase enzyme

2.3.6 Production of streptokinase enzyme.

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CHAPTER 3 MATERIALS AND METHOD

3.1 Materials

3.1.1 Substrates

3.1.2 Microbial strains

3.1.3 Reagents

3.1.4 Equipment

3.2 Methods

3.2.1 Screening of fungi and bacteria for production of therapeutic enzymes

3.2.2 Preparation of fermentation substrates

3.2.3 Pre-treatment of substrates

3.2.4 Solid-state fermentation and submerged fermentation

3.2.5 Extraction conditions

3.2.6 Assaying for therapeutic enzymes

3.2.7 Biomass monitoring

3.2.8 Optimization of conditions for production of therapeutic enzyme

CHAPTER 4 RESULT

CHAPTER 5 DISCUSSION

CHAPTER 6 CONCLUSION

CHAPTER 7 FUTURE PERSSPECTIVE CHAPTER 8 LITERATURE CITED

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